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# Mechanoreceptors on Neurotrophins, trk Receptors, and P75 LNGFR

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**The impact of null mutations of the genes for the NGF family of neurotrophins and their receptors was examined among the wide variety of medium to large caliber myelinated mechanoreceptors which have a highly specific predictable organization in the mystacial pad of mice. Immunofluorescence with anti-protein gene product 9.5, anti-200-kDa neurofilament protein (RT97), and anti-calcitonin gene-related product was used to label innervation in mystacial pads from mice with homozygous null mutations for nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), the three tyrosine kinase receptors (trkA, trkB, trkC), and the low-affinity nerve growth factor receptor p75. Specimens were sacrificed at birth and at 1, 2, and 4 weeks for each type of mutation as well as at 11 weeks and 1 year for p75 and trkC mutations, respectively. Our results demonstrate several major concepts about the role of neurotrophins in the development of cutaneous mechanoreceptors that are supplied by medium to large caliber myelinated afferents. First, each of the high-affinity tyrosine kinase receptors, trkA, trkB, and trkC, as well as the low-affinity p75 receptor has an impact on at least one type of mechanoreceptor. Second, consistent with the various affinities for particular trk receptors, the elimination of NGF, BDNF, and NT-3 has an impact comparable to or more complex than the absence of their most specific high-affinity receptors: trkA, trkB, and trkC, respectively. These complexities include potential NT-3 signaling through trkA and trkB to support some neuronal survival. Third, most types of afferents are dependent on a different combination of neurotrophins and receptors for their survival: reticular and transverse lanceolate afferents are dependent upon NT-3, NGF, and trkA; Ruffini afferents upon BDNF and trkB; longitudinal lanceolate afferents upon NGF, trkA, BDNF, and trkB; and Merkel afferents on NGF, trkA, NT-3, trkC, and p75. NT-4 has no obvious detrimental impact on the mechanoreceptor development in the presence of BDNF. Fourth, NT-4 and BDNF signaling through trkB may suppress Merkel innervation and NT-3 signaling through trkC may suppress Ruffini innervation. Finally, regardless of the neurotrophin/receptor dependency for afferent survival and neurite outgrowth, NT-3 has an impact on the formation of all the sensory endings. In the context of these findings, indications of competitive and suppressive interactions that appear to regulate the balance of innervation density among the various sets of innervation were evident.** 

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## INTRODUCTION

The nerve growth factor (NGF)-related family of neurotrophins has been shown to play an important role in the development, maintenance, and response to injury of the nervous system (for recent reviews see Bothwell, 1995; Davies, 1997; Lewin and Barde *et al.*, 1996; McMahon *et al.*, 1996; Segal and Greenberg,, 1996; Snider, 1994; Snider and Wright, 1996). The known members of this family in mammals are NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). All of these neurotrophins are expressed in target tissues of peripheral innervation as well as in or around the developing peripheral ganglia (Arumäe *et al.*, 1993; Buchman and Davies, 1993; ElShamy and Ernfors *et al.*, 1996a; Ernfors *et al.*, 1992; Fariñas, *et al.* 1996; Ibáñez *et al.*, 1993; Verdi *et al.*, 1996; Wilkinson *et al.*, 1996), where they are believed to control the survival of neurons and precursors and to influence regulation of axonal growth and branching and perhaps phenotypic differentiation.

The effects of these neurotrophins are presumably mediated through high-affinity binding with the tyrosine kinase (trk) family of receptors: trkA, trkB, and trkC. Each of these receptors has a specific extracellular domain that binds particular neurotrophins, which in turn activates a tyrosine kinase intracellular domain resulting in the biological responses. NGF signals specifically through trkA (Hempstead *et al.*, 1991; Kaplan *et al.*, 1991; Klein *et al.*, 1991a), whereas BDNF and, to a lesser degree, NT-4 signal through trkB (Glass *et al.*, 1991; Ip *et al.*, 1992; Klein *et al.*,1991b, 1992; Soppet *et al.*, 1991; Squinto *et al.*, 1991). NT-3 acts with high affinity through trkC (Lamballe *et al.*, 1991) and binds to a lesser degree through trkA and trkB (Cordon-Cardo *et al.*, 1991; Davies *et al.*, 1995; Ip *et al.*, 1993; Klein *et al.*, 1991b; Lamballe *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991). A low-affinity NGF receptor (p75) binds all members of the neurotrophin family (Dechant *et al.*, 1994), but its functional significance has been puzzling since it lacks a recognizable active intracellular domain. Proposals about its function range from enhanced activity in collaboration with the trk receptors to reduced activity by competing for neurotrophins or blocking their access to the trk receptors.

Mice with a homozygous null mutation of genes for a neurotrophin (NGF, BDNF, NT-3, or NT-4) or a high-affinity neurotrophin receptor (trkA, trkB, or trkC) have reduced survival of specific sets of sensory and/or sympathetic neurons (Conover *et al.*, 1995; Crowley *et al.*, 1994; Ernfors *et al.*, 1994a,b; Fariñas *et al.*, 1994; Jones *et al.*, 1994; Klein *et al.*,1993, 1994; Koliatsos *et al.*, 1994; Lee *et al.*, 1992; Liu *et al.*, 1995; Smeyne *et al.*, 1994; Tessarollo *et al.*, 1994). Based on the quantities and relative cell body sizes of lost neurons in each type of knockout, some neurons depend only on one neurotrophin for their survival, whereas others may depend on more than one. For example, the absence of NGF results in a loss of at least 70–80% of the dorsal root ganglion (DRG) and trigeminal ganglion neurons that would normally survive, whereas the absence of NT-3 results in a 60% loss (Crowley *et al.*, 1994; Ernfors *et al.*, 1994b; Fariñas *et al.*, 1994; Davis, unpublished). Virtually all of the smaller neurons require NGF and some of these need NT-3. Both NGF and NT-3 null mutations also have a loss of some larger neurons, indicating that multiple types of afferents are affected. Whereas the small neurons that are dependent on NGF include unmyelinated nociceptors (Lewin and Mendell, 1993), the identity of the larger NGF-dependent neurons is unknown as is the identity of neurons requiring both NGF and NT-3.

In some cases, the analyses of cell losses reveal discrepancies between mutants lacking a functional neurotrophin and those lacking the corresponding high-affinity receptor. For example, only a 10–20% increased cell loss occurs in the trigeminal ganglia of mice with homozygous null mutations of trkC, which is the high-affinity receptor for NT-3. Thus, the survival of some neurons appears to depend upon lower affinity binding to other receptors. Whereas muscle spindle afferents require both NT-3 and trkC (Ernfors *et al.*, 1994b; Fariñas *et al.*, 1994; Hohn *et al.*, 1990; Hory-Lee *et al.*, 1993; Klein *et al.*, 1994), the receptor dependencies are unknown for Merkel and D-hair afferents which also depend upon NT-3 (Airaksinen *et al.*, 1996).

Some of the overlaps and discrepancies among the cell survival data across various knockouts may be related to switches in trk receptor expressions during the development of many neurons (Buchman and Davies, 1993; Buj-Bello *et al.*, 1994; Davies *et al.*, 1995, 1997). Also, the neurotrophin dependencies may be related to developmental roles other than neuronal survival, such neurite outgrowth, branching, ending formation, and maintenance. To date, little is known about the roles of these neurotrophic interactions, particularly in relation to the wide variety of peripheral innervations.

The mystacial pad of the mouse trigeminal system is especially powerful for assessing such peripheral impacts because it contains a wide variety of innervation which is distributed to highly specific, predictable locations among vibrissal follicle sinus complexes (FSCs) and the intervibrissal fur. As seen in the mouse and rat, each FSC consists of a vibrissal follicle encased within an encapsulated vascular sinus and contains at least six different sets of myelinated cutaneous mechanoreceptors as well as a variety of unmyelinated peptidergic and nonpeptidergic sensory and autonomic nerve endings (Davis *et al.*, 1997; Fundin *et al.*, 1994; Rice *et al.*, 1993, 1997a). The innervation to the intervibrissal guard hairs consists of up to four types of presumptive mechanoreceptors as well as a variety of unmyelinated free nerve endings (Davis *et al.*, 1997; Fundin *et al.*, 1994, 1997; Rice *et al.*, 1993).

Because of this precise and predictable distribution of the various types of sensory and autonomic nerve endings, the mystacial pad of the mouse was used to examine the impact of homozygous null mutations (−/−) of a gene for either BDNF, NT-4, NT-3, or NGF or trkA, trkB, trkC, or p75 on mechanoreceptor innervation. Every type of mechanorecep-

tor was found to be affected by a combination of at least one neurotrophin and one receptor deletion. In some cases the impact was on the existence of both the axons and endings and in other cases just the endings.

## MATERIALS AND METHODS

### Animals

Mice were obtained from overnight mating of (1) trkA$^{+/-}$ (Smeyne *et al.,* 1994), (2) trkB$^{+/-}$ (Klein *et al.,* 1993), (3) trkC$^{+/-}$ (Klein *et al.,* 1994), (4) p75$^{-/-}$ (Lee *et al.,* 1992), (5) NGF$^{+/-}$ (Crowley *et al.,* 1994), (6) BDNF$^{+/-}$ (Ernfors *et al.,* 1994a), (7) NT-4$^{+/-}$ (Liu *et al.,* 1995), or (8) NT-3$^{+/-}$ mice (Ernfors *et al.,* 1994b). The mice were genotyped by polymerase chain reaction. Only homozygous mutant mice and wild-type mice were studied. For each neurotrophin or receptor, at least five knockouts and five wild-type mice were selected at birth (P0), 1 week (P4–P6), and 2 weeks (P12–P16) postnatally. Older knockouts and wild-type mice (P25, P28, P31, 11-week-, or 1-year-old mice) were included for trkA, trkC, p75(LNGFR), BDNF, NGF, and NT-4.

Most of the mice were overdosed with chloral hydrate or sodium pentobarbital and perfused transcardially first with a solution of 0.9% sodium chloride at room temperature and then with cold 4% paraformaldehyde in 0.1 *M* PBS at pH 7.4. Some newborn specimens were anesthetized by hypothermia and the mystacial pads were removed and fixed by immersion in 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4.

### Tissue Preparation

The mystacial pads were removed and postfixed at 4°C in the perfusion fixative for 1 hr, rinsed in PBS, and cryprotected by overnight infiltration with 20% sucrose in PBS. Fourteen-micrometer-thick sections were cut on a cryostat perpendicular to the skin surface and parallel to the rows of follicles. This resulted in sections oriented approximately along the length of the FSCs. Some specimens were cut parallel to the skin surface, resulting in cross sections of the FSCs. The sections were directly mounted onto slides coated with chrome-alum gelatin and air dried.

### Immunofluorescence

Immunofluorescence analyses were performed using a polyclonal antibody against a pan-neuronal cytoplasmic antigen, protein gene product 9.5 (PGP 9.5) (1:2000, Ultraclone Ltd.), which labels all known neuronal structures in the skin (Rice *et al.,* 1993). A monoclonal antibody, RT97 (1:250, gift from Dr. J. Wood [Wood, 1981]; 1:500, Peninsula, Inc.), against a 200-kDa phosphorylated neurofilament protein was used which generally labels cell bodies, axons, and most endings of myelinated neurons (Fundin *et al.,* 1997; Rice *et al.,* 1997a; Robertson and Grant, 1989). Sections primarily for another study were also prepared with a rabbit polyclonal antibody against calcitonin gene-related peptide (CGRP) (1:800, Peninsula Inc.), which is a broadly distributed neuropeptide. The sections were first preincubated with bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS for 1 hr and then incubated with a solution of primary antibody overnight at 4°C at high humidity. The primary antibody was diluted in PBS with 1% BSA and 0.3% Triton X-100. Following the primary incubation, the slides were rinsed in excess PBS for $\frac{1}{2}$ hour and then incubated at room temperature for 1–2 hr with secondary antibodies diluted in PBS with 1% BSA with 0.3% Triton X-100. To detect polyclonal primary antibodies (i.e., anti-PGP 9.5, anti-CGRP), donkey anti-rabbit conjugated to cyanine-3.18 (Cy3) (1:500; Jackson Immuno Research Laboratories, Inc.) or swine anti-rabbit conjugated to tetramethylrhodamine isothiocyanate (TRITC) (1:40; DAKO A/S) secondary antisera was used. To detect the monoclonal primary antibody RT97, rabbit anti-mouse conjugated to TRITC or fluoroscein isothiocyanate (FITC) (1:40, DAKO A/S) or donkey anti-mouse conjugated to FITC (1:50, Jackson Immuno Research Laboratories, Inc.) was used. The slides were then rinsed in excess PBS and mounted with 0.1% paraphenylene diamine (PPD) in glycerol.

Double labeling with the polyclonal PGP 9.5 and the monoclonal RT97 primary antibodies was performed by first incubating with one of the desired primary antibodies followed by the appropriate TRITC-conjugated secondary antibody and then incubating with the other primary antibody followed by its appropriate FITC-labeled secondary antibody.

### Analysis

Sections were viewed and photographed with a Nikon Microphot FX equipped for TRITC and FITC epifluorescence. T-MAX 400 ASA and Technical Pan negative film at 400 ASA were routinely used. The qualitative analysis was based on 70 mutant and 50 wild-type mice from which both sides of the mystacial pad were processed, analyzed, and compared. All five rows (A–E) of vibrissal FSCs on each side were included in the as well as the larger $\alpha$, $\beta$, $\delta$, and $\gamma$ FSCs that straddle the caudal ends of the rows.

## RESULTS

### Normal Organization of Mechanoreceptors in the Mystacial Pad

The basic structure and mechanoreceptor innervation of vibrissal FSCs and guard hair follicles is shown in Fig. 1. Examples of this innervation are shown by immunofluorescence throughout Figs. 3 and 5–8. All of the photomicrographs are of anti-PGP 9.5 labeling except for those labeled with RT97, which is indicated in the lower right hand corner. Immunofluorescence with both anti-PGP 9.5 and RT97 revealed large and medium caliber myelinated axons that entered vibrissal FSCs via a deep vibrissal nerve (DVN). These axons gave rise to four types of presumptive low-threshold mechanoreceptors located in deeper targets (Figs. 1, 3–6, and 8). Superficial vibrissal nerves (SVN) supplied medium caliber myelinated axons that formed two types of mechanoreceptors in superficial targets of FSCs (Figs. 1 and 3–6). Small skin nerves supplied medium caliber axons that formed four types of mechanoreceptors affiliated with intervibrissal guard hair follicles (Figs. 1, 5, and 7).

As shown in Fig. 1, both the DVN and SVNs were sources of vibrissa-related Merkel afferents that respectively had large and medium caliber axons. Merkel afferents were the only ones whose axons crossed the basement membrane and formed endings on epidermally derived targets, the Merkel cells. The combinations of Merkel endings and cells are referred to as Merkel cell–neurite complexes (Munger and Ide, 1988). The Merkel innervation from the DVNs terminated in the outer root sheath at the level of the ring sinus

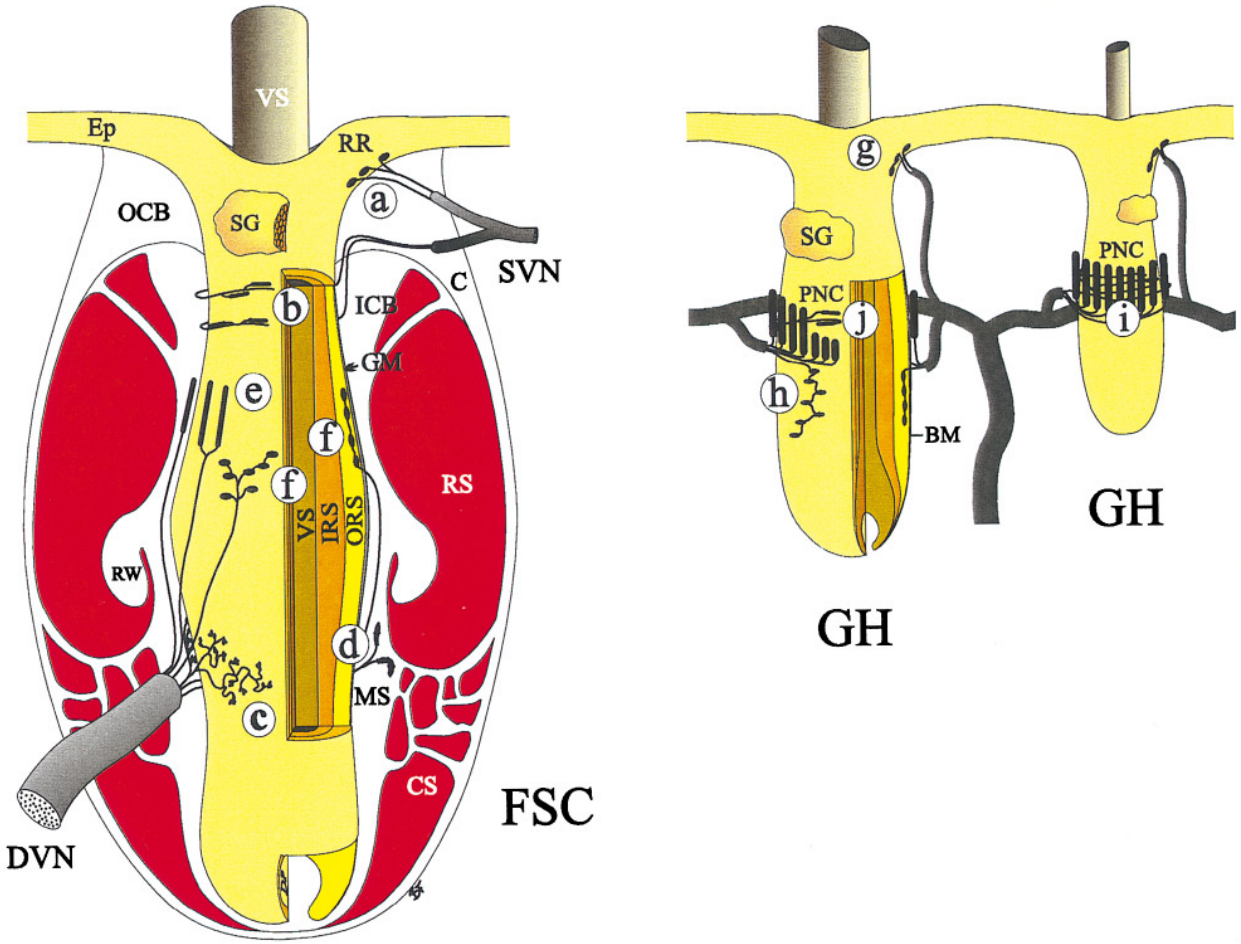



**FIG. 1.** Schematic cutaway drawing of a vibrissal follicle–sinus complex (FSC) and intervibrissal guard hair follicles (GH) illustrating the structural components and their mechanoreceptor innervation (see Fundin *et al.*, 1997, and Rice *et al.*, 1997a, for details). The follicles are derived from the epidermis (Ep) and are composed of an inner and an outer root sheath (IRS and ORS) surrounded by a basement membrane (BM). The basement membrane is especially thick around the vibrissal follicle and is referred to as the glassy membrane (GM). In the FSC, the vibrissal follicle (light yellow) is suspended within a dermally derived vascular sinus (red) which is enclosed within a dense collagen capsule (C). The sinus has two distinct zones: the cavernous sinus (CS), which is partially filled with trabeculae, and the ring sinus (RS), which has an open lumen. The glassy membrane is lined by a loose connective tissue layer referred to as the mesenchymal sheath (MS), which expands at the apex of the sinus to form the inner conical body (ICB). A donut-shaped structure, the ringwulst (RW) is suspended around the circumference of the mesenchymal sheath at the level of the ring sinus. The epidermis at the mouth of the FSC is especially thick and is referred to as the rete ridge collar (RR). Each FSC is innervated by a relatively large deep vibrissal nerve (DVN) and several tiny superficial vibrissal nerves (SVN). Both sets of nerves are accompanied by small arterioles. Two sets of mechanoreceptor are supplied by the SVNs: (a) Merkel endings in the epidermis at the mouth of the FSC and (b) transverse lanceolate endings in the inner conical body. The DVN supplies four sets of mechanoreceptors: (c) reticular endings and (d) Ruffini endings in the mesenchymal sheath at the upper level of cavernous sinus; (e) longitudinal lanceolate endings in the mesenchymal sheath at the level of ring sinus; (f) Merkel endings in the outer root sheath at the upper level of the ring sinus. The innervation to the guard hairs arises from small skin nerves that form a four tiered dermal plexus. At the mid follicular level, guard hair follicles are surrounded by a piloneural complex which contains a mixture of endings from myelinated and unmyelinated axons. All of the A$\beta$ myelinated axons come from the second tier and these supply all four types of mechanoreceptors that can be affiliated with guard hair follicles: (g) Merkel endings in the epidermis at the mouth of the follicles; (h) Merkel endings in the outer root sheath below the level of piloneural complexes affiliated with the largest guard hairs; (i) a palisade of longitudinally oriented lanceolate endings in the piloneural complex; (j) transverse lanceolate endings in the piloneural complex. Sebaceous gland (SG).

of the vibrissal follicle (Fig. 1, f). Those supplied by the SVNs were situated in the basal layer of the rete ridge collar, which is the thickened epidermis at the mouth of the vibrissal FSCs (Fig. 1, a). In the mouse, anti-PGP 9.5 labeled the axons, endings, and Merkel cells, RT97 labeled just the axons, and anti-CGRP labeled just the Merkel cells.

The other three mechanoreceptors supplied by medium to large caliber myelinated DVN axons were located in the dermally derived mesenchymal sheath that lines the thick basement membrane (''glassy membrane'') of the vibrissal follicle within each FSC (Fig. 1). These mechanoreceptors were (1) reticular endings (Fig. 1, c), (2) Ruffini endings (Fig. 1, d), and (3) longitudinally oriented lanceolate endings (Fig. 1, e). The longitudinal lanceolate endings formed a palisade around the vibrissal follicle. The axons and endings of these three mechanoreceptors labeled with anti-PGP 9.5. RT97 immunoreactivity was present in all of their axons but was only in the Ruffini and lanceolate endings.

In addition to the Merkel innervation at the mouth of the FSCs, the second type of mechanoreceptor supplied by the SVNs was a set of thinner lanceolate endings (Fig. 1, b). These arose *en passant* and were oriented transversely around the neck of the follicle where the mesenchymal sheath expands to form the inner conical body (ICB). The SVNs also supplied the inner conical body with a dense circumferentially array of unmyelinated innervation. Both the transverse lanceolate and the unmyelinated innervation labeled with anti-PGP 9.5 but only the lanceolate labeled with RT97.

In the intervibrissal fur, a dermal plexus supplied Merkel innervation to the epidermis at the mouth (Fig. 1, g) of many guard hair follicles. At a mid-follicle level, most guard hair follicles were innervated by piloneural complexes (Biemesderfer *et al.*, 1978) which consisted of a palisade of longitudinally oriented lanceolate endings (Fig. 1, i), a few transverse lanceolate endings (Fig. 1, j), and numerous circumferentially oriented free nerve endings (Fundin *et al.*, 1997). Some larger guard hair follicles, especially near the nose and upper lip, had an additional set of Merkel innervation (Fig. 1, h) in the outer root sheath below the level of the piloneural complex.

In wild-type mice, most Merkel cells were present and innervated on the day of birth in all of their normal adult locations. The other types of mechanoreceptors described above either were lacking or were immature. After the first postnatal week, all of these other mechanoreceptors were evident and more mature. By 2 weeks postnatally, all of the innervation was similar to that in the adult.

All mechanoreceptors studied responded differently to the various neurotrophin and neurotrophin receptor null mutations. Moreover, all types of sensory nerve endings studied required a specific neurotrophin or a combined set of neurotrophins for their normal development and/or maintenance. The results from the different null mutations are summarized in Fig. 9.

### *p75(LNGFR) Involvement in Neurofilament Expression during Development*

The absence of p75 resulted in a transient loss of RT97 expression in the distal portions of DVN mechanoreceptor afferents. Throughout the postnatal development of wild-type mice, RT97-IR was expressed in the axons of all mechanoreceptor afferents and all their endings except the reticular endings. On the day of birth in the $p75^{-/-}$ mice, all vibrissal mechanoreceptor axons were completely labeled with RT97. However, in 1- and 2-week-old $p75^{-/-}$ mice, RT97-IR was only expressed in DVN axons considerably proximal to their entry into the FSCs (Fig. 2). In contrast, SVN axons still showed weak RT97-IR throughout their length. Four weeks postnatally, the RT97-LIR was restored to the distal end of the DVN mechanoreceptor axons.

### *Merkel Ending Dependency on NGF, NT-3, trkA, trkC, and p75*

Anti-PGP 9.5 not only labeled the Merkel axons and endings but also the Merkel cells (Figs. 3–5). RT97 labeled just the axons and endings. CGRP immunoreactivity was expressed in the Merkel cells but not the axons or terminals. All sets of Merkel innervation (i.e., in the outer root sheath of vibrissal FSCs and at the mouths of vibrissal and guard hair follicles) were eliminated during development in $NT\text{-}3^{-/-}$ and $trkC^{-/-}$ mice, although the loss occurred earlier in the absence of NT-3. Some Merkel afferents were also eliminated in NGF and trkA knockouts. All sets were gradually reduced over several months in $p75^{-/-}$ mutants. Surprisingly, Merkel innervation increased in the absence of NT-4, BDNF, trkB, and, in some locations, NGF.

In newborn $trkC^{-/-}$ mice, the Merkel innervation throughout the mystacial pad appeared to be fairly normal (not shown). By P6, a decrease in the number of Merkel cells was evident in all locations and only a few were innervated (Figs. 3C and 3D). At the end of 2 postnatal weeks, only a few uninnervated Merkel cells were evident in any of the expected locations (large arrow in Fig. 3E). These were completely absent in 4-week- and 1-year-old $trkC^{-/-}$ mice.

In the absence of Merkel cells and endings, some large caliber axons ascended from the DVN to the inner conical body in 1-week and older $trkC^{-/-}$ mice. Normally the DVNs only supply a few unmyelinated axons to the level of the inner conical body, which is innervated almost entirely from SVNs.

In contrast to the gradual impact of trkC elimination, the absence of its ligand NT-3 resulted in an earlier more detrimental effect on Merkel innervation. At birth, numerous Merkel cells were present in all appropriate locations (Fig. 3G) but these were rarely innervated. By P5, only a few Merkel cells could be faintly labeled (large arrow in Fig. 3H) and none of these were innervated. Two weeks postnatally, neither Merkel cells nor endings were detected (Fig. 3I). In contrast to the trkC knockouts, considerably more large caliber axons ascended abnormally from the DVNs to the inner conical body (Fig. 3I, see also Fig. 6E), where they clearly joined the circumferentially oriented endings from the SVNs.

Unlike the global, early loss of Merkel cell–neurite complexes caused by the absence of NT-3 or trkC, the elimination of p75 had a later effect that varied among different locations. First, a substantial increase of Merkel cells was

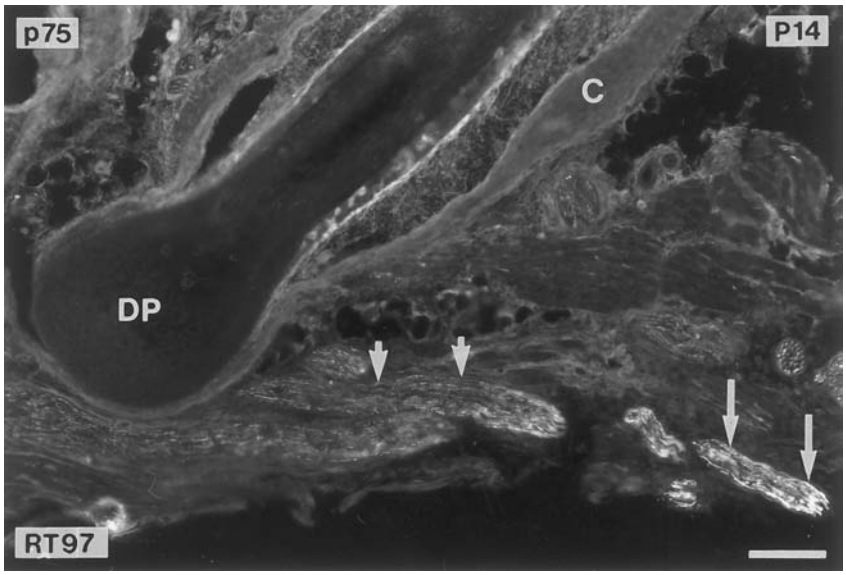



**FIG. 2.** Immunofluorescence photomicrograph showing RT97 immunoreactivity at the level of the deep end of vibrissal FSCs in a 2-week-old $p75^{-/-}$ mouse. Nerve fascicles from a row nerve carry axons that show a decline in their RT97-IR in a caudal (long arrows) to rostral (short arrows) direction. Scale bar, 30 $\mu$m.

evident at the mouth of the vibrissal FSCs (small arrows in Figs. 4A and 4B) and guard hair follicles during the first 2 postnatal weeks. However, only a small proportion of those cells at the mouth of the FSCs—perhaps no more cells than normal—were clearly contacted by Merkel endings. Four-week-old $p75^{-/-}$ mice had a noticeable decrease in the number of terminals and detectable Merkel cells at the mouths of FSCs and guard hair follicles. By the 11th week, only a few Merkel cells could be detected faintly with anti-PGP 9.5, of which only a few were definitely innervated (not shown).

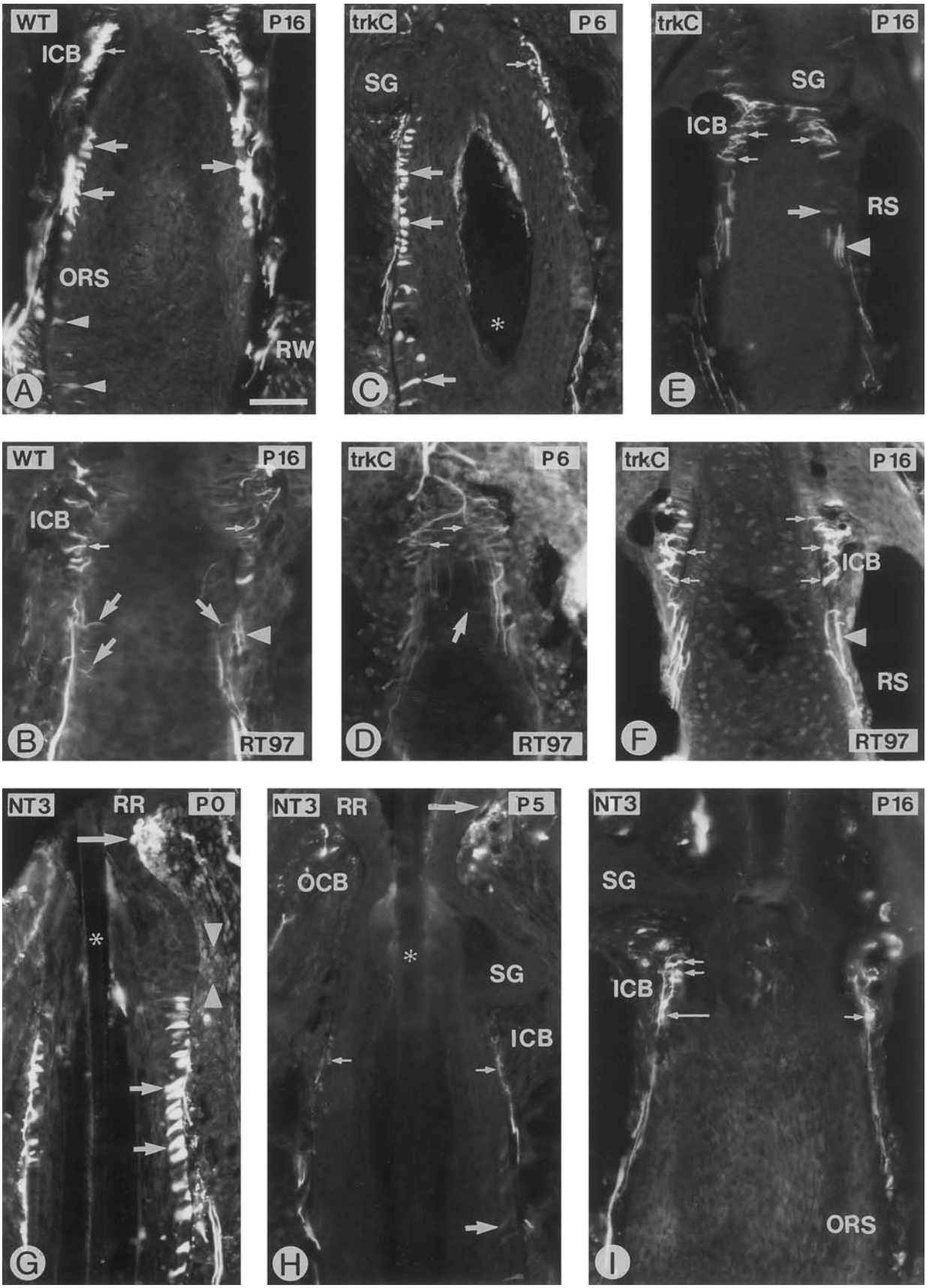
At the level of the ring sinus in vibrissal FSCs (Fig. 1), the Merkel innervation appeared normal in $p75^{-/-}$ mice at birth. Unfortunately, a clear evaluation of Merkel cell innervation at this location could not be done on P7 and P14 in $p75^{-/-}$ mice because of the lack of RT97-IR in their terminals and distal axons (Fig. 2). However, no obvious decrease was detected among the Merkel cells nor the apparent innervation as revealed by anti-PGP 9.5 (large arrows in Figs. 4A and 4B). In 4-week-old $p75^{-/-}$ mice, a substantial reduction in the number of Merkel endings had occurred, but the Merkel cells were not obviously decreased. In 11-week-old $P75^{-/-}$ mice only occasional weakly labeled terminals were detected with anti-RT97 (large arrow in Fig. 4D), even though only a slight if any decrease had occurred in the number of detectable Merkel cells (large arrows in Fig. 4C). However, the Merkel cells labeled only faintly with anti-PGP and anti-CGRP and they were large, with a rounded shape (Fig. 4C), instead of a somewhat flattened shape, as in the wild-type mice.

In contrast to the losses incurred in NT-3, trkC, and eventually p75 null mutants, the $NT\text{-}4^{-/-}$, $BDNF^{-/-}$, and $trkB^{-/-}$ mice appeared to have an increase in Merkel cell–neurite complexes, especially at the mouths of vibrissal FSCs and guard hairs (Figs. 5B, 5D, and 7B). Also, the proportion of guard hair follicles with Merkel innervation increased from less than half to nearly 100% (Figs. 5B and 7B). In the outer root sheath of the vibrissal FSCs, the Merkel cell–neurite complexes of NT-4, BDNF, and trkB knockouts were more widespread than normal. In wild-type mice, the Merkel cell–neurite complexes were normally distributed to a level just at the upper end of the ring sinus, above the level of a donut-shaped structure referred to as the ringwulst (Fig. 3A). In the NT-4, BDNF, and trkB knockouts, the Merkel cell–neurite complexes were distributed at levels throughout the level of the ring sinus and continued down to the level of the cavernous sinus well below the ringwulst (Figs. 5D and 8D).

Similar increases of Merkel cells were also obvious in the intervibrissal fur (Fig. 5C) after NGF null mutations but less so at the mouths of the vibrissal FSCs. Such an increase was not evident in $trkA^{-/-}$ mice. However, in contrast to increases in NT-4, BDNF, and trkB null mutants, the Merkel cell–neurite complexes in the outer root sheath of FSCs in both $NGF^{-/-}$ and $trkA^{-/-}$ mice were reduced and closely packed at the uppermost levels of the ring sinus (large arrows in Fig. 5E). The lower portion of the ring sinus was abnormally devoid of Merkel cell–neurite complexes.

### *Longitudinal Lanceolate Ending Dependency on NGF, BDNF, trkA, and trkB*

At the level of the ring sinus in vibrissal FSCs (Fig. 6a), some longitudinal lanceolate afferents terminated as one


WT
P16
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ORS
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or two long endings (long arrows), whereas others formed several short endings (short arrows). In general the longer, less branched endings were distributed higher up the mesenchymal sheath nearer the inner conical body, whereas the shorter more branched endings were located deeper near the ringwulst. None of the neurotrophin or receptor null mutant mice showed a complete loss of longitudinal lanceolate endings; however, different deficits were observed in the absence of BDNF and trkB compared to NGF and trkA. The loss of NT-3 but not trkC had an impact on ending morphology.

In 1-week-old BDNF$^{-/-}$ and trkB$^{-/-}$ mice, an obvious decrease in the number of longitudinal lanceolate endings in the FSCs was evident (Fig. 6C), which persisted through the later ages examined (i.e., up to P25). The few remaining longitudinal lanceolate endings, especially in trkB$^{-/-}$ mice, were thin and long and emanated from axons with very few branches (Fig. 6C). Although most of the longitudinal lanceolate endings were eliminated in the absence BDNF or trkB, lack of NT-4 had no obvious effect.

TrkA null mutations also resulted in a reduction of longitudinal lanceolate endings (Fig. 6B) but this was not as severe as in the trkB$^{-/-}$ and BDNF$^{-/-}$ mice. In contrast to the remaining endings in trkB$^{-/-}$ and BDNF$^{-/-}$ mice, those present in the absence trkA were short and thick and emanated from axons with more branches (large arrow in Fig. 6B). Interestingly, the loss of NGF also resulted in a decrease in longitudinal lanceolate endings but not as much as in the absence of trkA. NGF$^{-/-}$ mice also had both types of branching patterns. Between the next 1 and 4 weeks, however, the longitudinal lanceolate endings in the NGF$^{-/-}$ mice gradually declined and became more comparable to those in the absence of trkA.

NT-3 null mutations did not noticeably affect the presence of longitudinal lanceolate endings but did alter their morphology. In the upper portion of the mesenchymal sheath, the branches of lanceolate afferents had a more transverse orientation and wider distribution, like the branches of Merkel afferents, although the endings still arose in a longitudinal orientation (arrows in Figs. 6E and 6F). However, the ending morphologies varied from an abnormal round disk shape to a more normal elongated lanceolate shape. Overall, the individual endings were broader than normal and had irregular margins that gave them a fuzzy appearance (Fig. 6F).

As in the vibrissal FSCs, no null mutation completely eliminated the palisades of longitudinal lanceolate endings in the piloneural complexes of intervibrissal guard hairs (Fig. 7). In wild-type mice, piloneural complexes were not present at birth and began to appear only gradually near the end of the first postnatal week (arrowheads in Figs. 5A and 5B). From the onset of the appearance of piloneural complexes, they were present on considerably fewer than normal guard hairs in BDNF$^{-/-}$ and trkB$^{-/-}$ mice at all ages studied (Fig. 7B). Moreover, each of these piloneural complexes had fewer longitudinal lanceolate endings than normal, rather than a complete palisade. In contrast, after trkA or NGF null mutations (not shown) hardly any guard hairs were innervated but the few palisades of longitudinal lanceolate endings were fairly complete.

### *Ruffini Ending Dependency on BDNF and trkB, but Not NT-4*

Deficits in Ruffini innervation were seen only in BDNF and, more severely, in trkB null mutants. An increase in endings occurred in the absence of NT-3 and, to a lesser degree, trkC.

During the first 2 postnatal weeks in wild-type mice, several large myelinated axons from DVNs gave rise to Ruffini endings located in the mesenchymal sheath at the level of the cavernous sinus of the vibrissal FSCs (insert in Fig. 8A).

**FIG. 3.** Immunofluorescence photomicrographs of FSCs showing the impact of trkC and NT-3 homozygous null mutations on Merkel innervation and transverse lanceolate innervation in vibrissal FSCs. The type of mutation is shown in the upper left corner; the postnatal age in the upper right corner. Sebaceous gland (SG), hair shaft (asterisk). Scale bar, 50 $\mu$m. (A,B) In FSCs of 2-week-old wild-type mice, anti-PGP 9.5 (A) labels the Merkel cells and their innervation (large arrows) in the outer root sheath at the level of the ring sinus (RS). A few faint Merkel cells (arrowheads) without innervation were located in the lower portion of the ring sinus at the level of the ringwulst (RW). In contrast, RT97 (B) labels the Merkel endings (large arrows) but not the Merkel cells. In the plane of section shown in B, RT97 also labels longitudinal lanceolate endings (arrowhead) at the level of the ring sinus as well as transverse lanceolate endings (small arrows) in the inner conical body (ICB). (C,D) One-week-old trkC$^{-/-}$ mice. Several Merkel cells (large arrows in C) were still labeled with anti-PGP, but only a few were affiliated with Merkel endings (large arrow in D) labeled with RT97. (E, F) In a 2-week-old trkC$^{-/-}$ mouse, only a few Merkel cells (large arrow in E) were faintly labeled with anti-PGP 9.5 but none were innervated (F) as seen with RT97. However, an increase occurred in transversely oriented RT97-positive profiles (small arrows in E and F) in the ICB, while a decrease occurred among the small caliber RT97 negative profiles. (G) The Merkel innervation in the NT-3$^{-/-}$ mouse at birth was comparable to that in the trkC$^{-/-}$ mouse at 1 postnatal week (C). Several Merkel cells were evident in the outer root sheath (short arrows) as well as at the rete ridge collar (RR, long arrow) at birth, but only occasional Merkel endings were detected by RT97 (not shown). As is also the case in normal newborn mice, the inner conical body lacks innervation (between arrowheads). (H) A week after birth in trkC$^{-/-}$ mice, only a few faint Merkel cells could be detected in the outer root sheath (large, short arrow) or in the rete ridge collar (large, long arrow). No Merkel endings were evident. (I) Anti-PGP 9.5 did not reveal any Merkel cells 2 weeks postnatally in NT-3$^{-/-}$ mice. However, several large caliber profiles ascended abnormally to the inner conical body (small, long arrow).

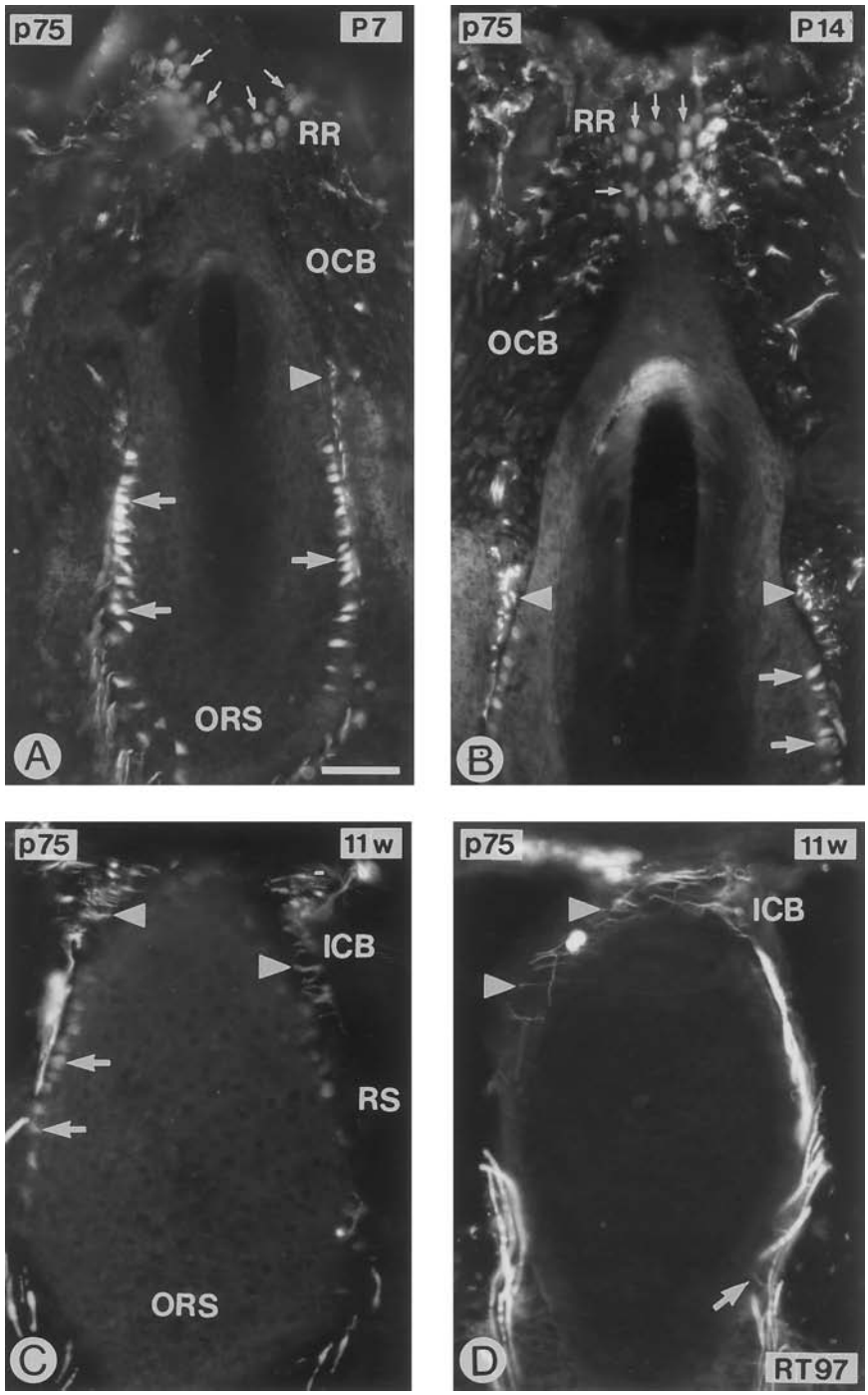



**FIG. 4.** Fluorescence photomicrographs showing the impact of p75 null mutations on Merkel innervation in the outer root sheath (ORS) and rete ridge collar (RR) of FSCs. The transverse lanceolate endings (arrowheads) in the inner conical body (ICB) were not affected. Scale bar, 50 $\mu$m. (A, B) In 1- and 2-week-old $p75^{-/-}$ mice, a dramatic increase in the number of Merkel cells (small arrows) was evident at the rete ridge collar, whereas the number of Merkel cells (large arrows) in the outer root sheath was not obviously changed. (C,D) Eleven weeks postnatally, no obvious decrease was evident among the Merkel cells in outer root sheath; however, they were faintly labeled with anti-PGP 9.5 (arrows in C). At this age, RT97 only revealed a few Merkel endings (large arrow in D) in the outer root sheath and only rare endings at the rete ridge collar.

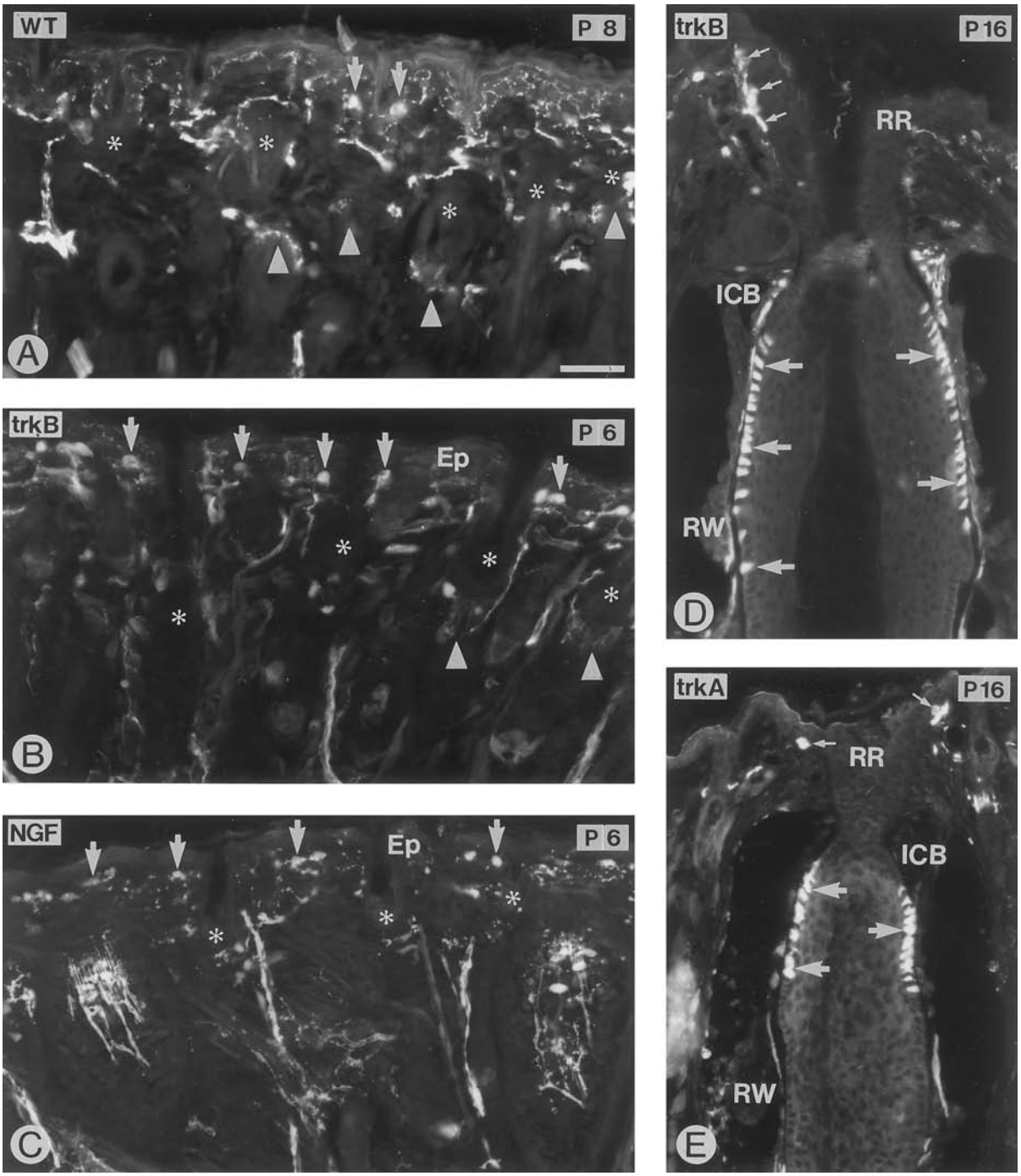



**FIG. 5.** Photomicrographs showing the increase in Merkel cell–neurite complexes in the absence of NGF, trkA, or trkB revealed by anti-PGP 9.5. Scale bar, 50 $\mu$m (A) Rostral mystacial pad in 1-week-old wild-type mice. Only a few of the guard hairs (asterisks) have Merkel innervation at the mouth of their follicles (arrows). Note the immature piloneural complexes (arrowheads). (B) One-week-old trkB$^{-/-}$ mice. A dramatic increase occurred in the number of guard hairs (asterisks) with Merkel cell–neurite complexes (arrows). Note the immature piloneural complexes (arrowheads). (C) A similar increase of Merkel cell–neurite complexes (arrows) was also detected in the 1-week-old NGF$^{-/-}$ mice. (D) In 2-week-old trkB$^{-/-}$ mice, Merkel cell–neurite complexes (large arrows) increased in the outer root sheath and abnormally expanded well below the level of the ringwulst (RW). Small arrows indicate still another distinct increase in the Merkel innervation at the rete ridge collar (RR). (E) Two-week-old trkA$^{-/-}$ mouse. In contrast to the expansion seen in trkB$^{-/-}$ mice (D), Merkel cell neurite complexes were abnormally tightly packed in the upper portion of outer root sheath (large arrows).

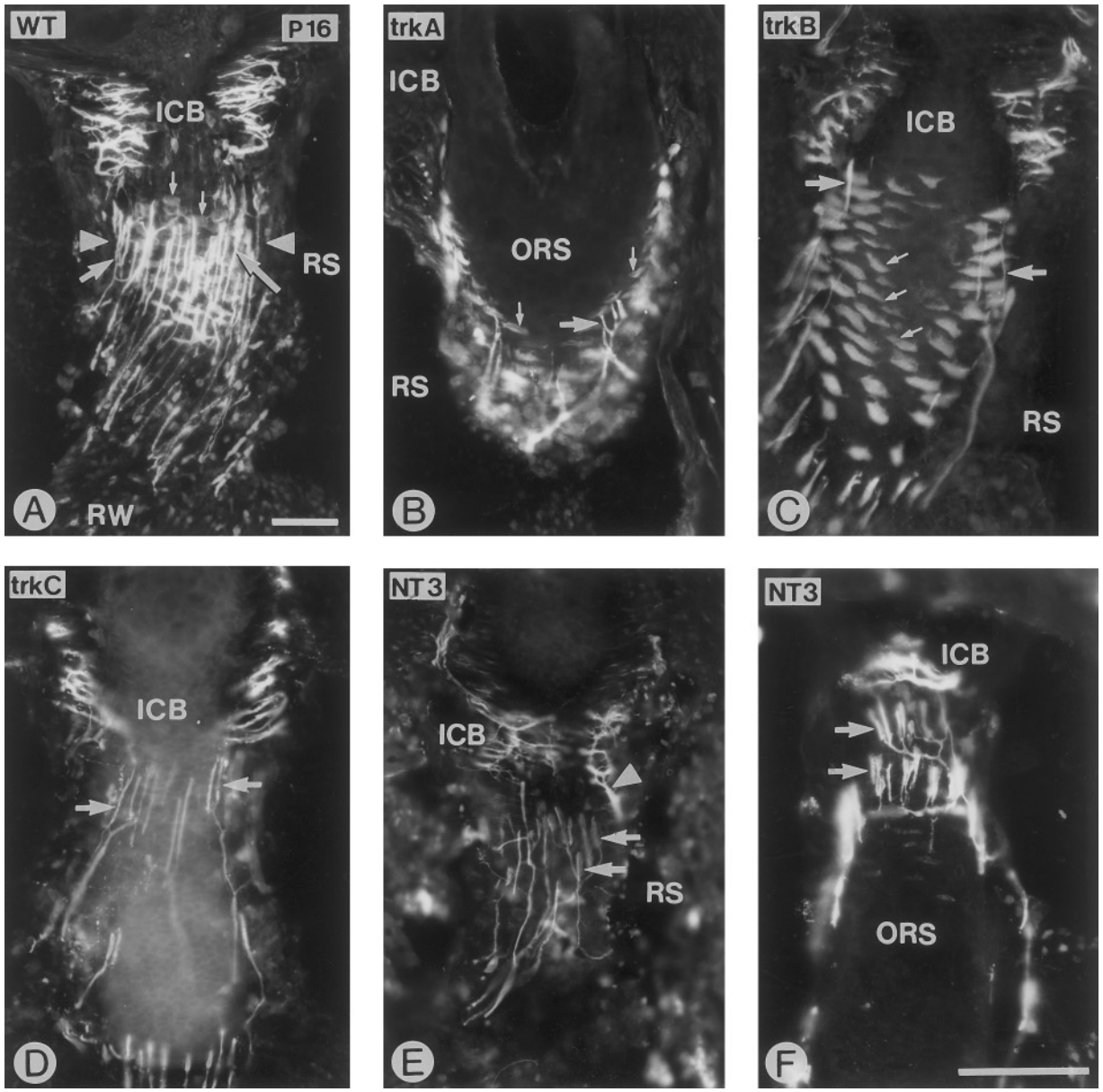



**FIG. 6.** Photomicrographs of the impact of various null mutations on longitudinal lanceolate endings and Merkel endings in FSCs as well as the innervation to the inner conical body (ICB) as labeled with anti-PGP 9.5 in 2-week-old mice. Small arrows indicate Merkel cells. Scale bar, 50 $\mu$m. (A) As seen in a wild-type mouse, a palisade of longitudinal lanceolate endings (between arrowheads) was present in the mesenchymal sheath above the ringwulst (RW). Two distinct types lanceolate afferents were evident: (1) those with several short endings (short arrows) and (2) those with one or two long endings (long arrows). In the inner conical body, note the dense circumferentially oriented innervation consisting of transverse lanceolate endings and numerous small and fine caliber unmyelinated axons and free nerve endings. (B) An oblique cut through the upper portion of the ring sinus in a trkA$^{-/-}$ mouse showing a substantial decrease in the number of longitudinal lanceolate endings. The few remaining endings are preferentially well branched and short (large arrow). Note the complete absence of innervation in the inner conical body. (C) In a trkB$^{-/-}$ mouse, a substantial decrease was evident among the longitudinal lanceolate endings in the FSCs. The few remaining endings are preferentially long and less branched (large arrows). Note the expansion of Merkel cell–neurite complexes in the outer root sheath (small arrows). (D) Lack of trkC did not obviously affect the longitudinal lanceolate endings (arrows). Note the absence of Merkel cell–neurite complexes. (E, F) Longitudinal lanceolate endings in NT-3$^{-/-}$ mice were not obviously reduced in number, but were abnormally short and highly branched in an irregular pattern (arrows). Rather than maintaining a longitudinal trajectory, the lanceolate afferents often turned in a transverse orientation, with a branching pattern similar to that of Merkel afferents, but the actual lanceolate endings were still oriented longitudinally. Also note the fuzzy appearing edges on the lanceolate endings, similar to that seen on the Ruffini endings in the cavernous sinus. An abnormally high number of large caliber axons (arrowhead in E) ascended from the DVN to the inner conical body where they joined the circumferentially oriented innervation.

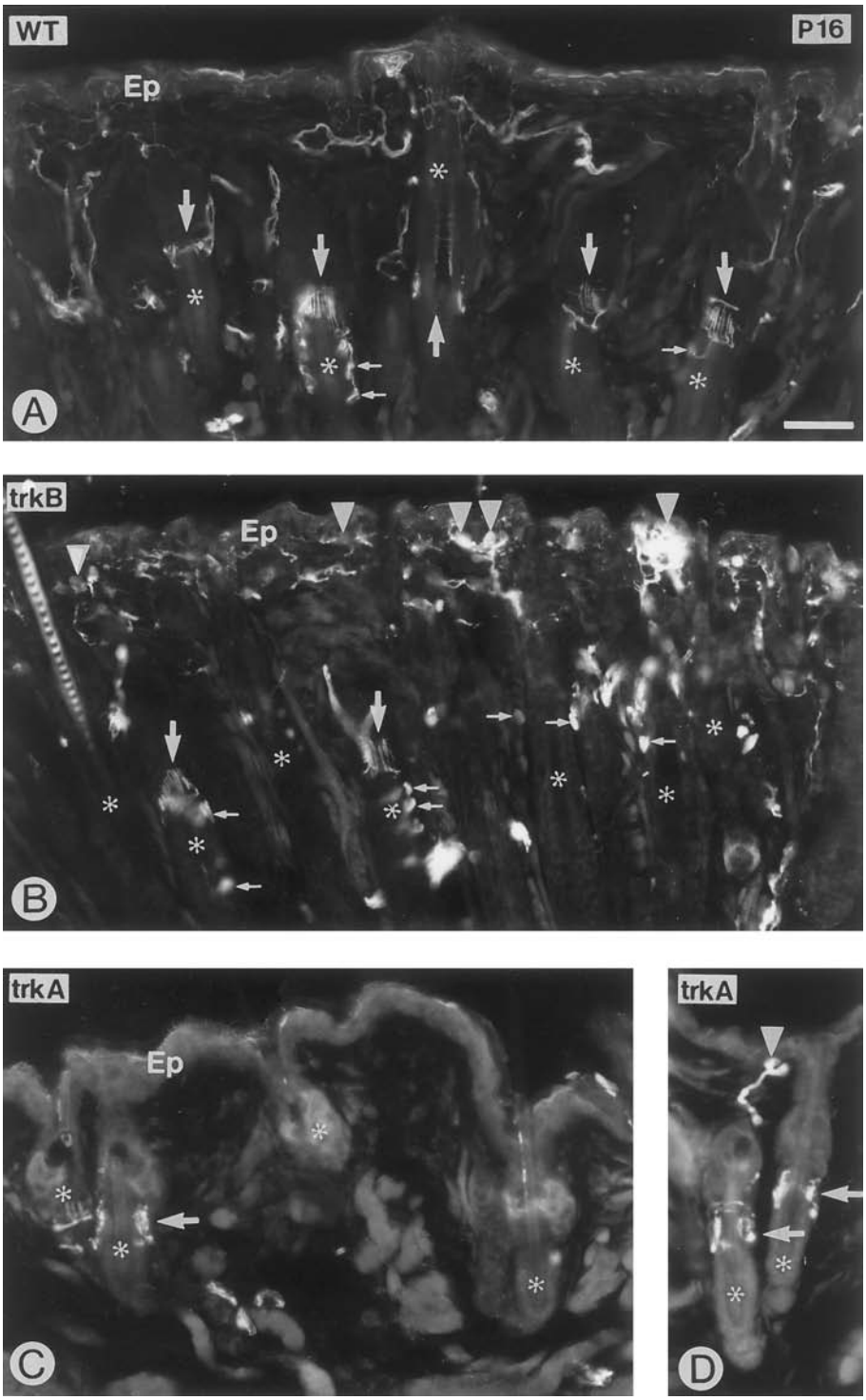



**FIG. 7.** Photomicrographs showing the decrease in the number of guard hair piloneural complexes in the absence of trkA and trkB as seen with anti-PGP 9.5 in 2-week-old mice. Guard hair follicles are indicated with asterisks. Ep, epidermis. Scale bar, 50 $\mu$m. (A) In the wild-type mouse, many guard hair follicles are innervated by piloneural complexes (large arrows). A few have Merkel endings at the mouth (not shown). The larger follicles also contain Merkel endings below the piloneural complex (small arrows). (B) In a trkB$^{-/-}$ mouse, a substantial decrease occurred in the number of guard hairs with piloneural complexes (large arrows). In contrast, note the dramatic increase in the number of Merkel cell–neurite complexes at the mouth (arrowheads) as well as below the piloneural complex (small arrows) of guard hairs. (C,D) Piloneural complexes (arrows) were drastically reduced in trkA null mutations.

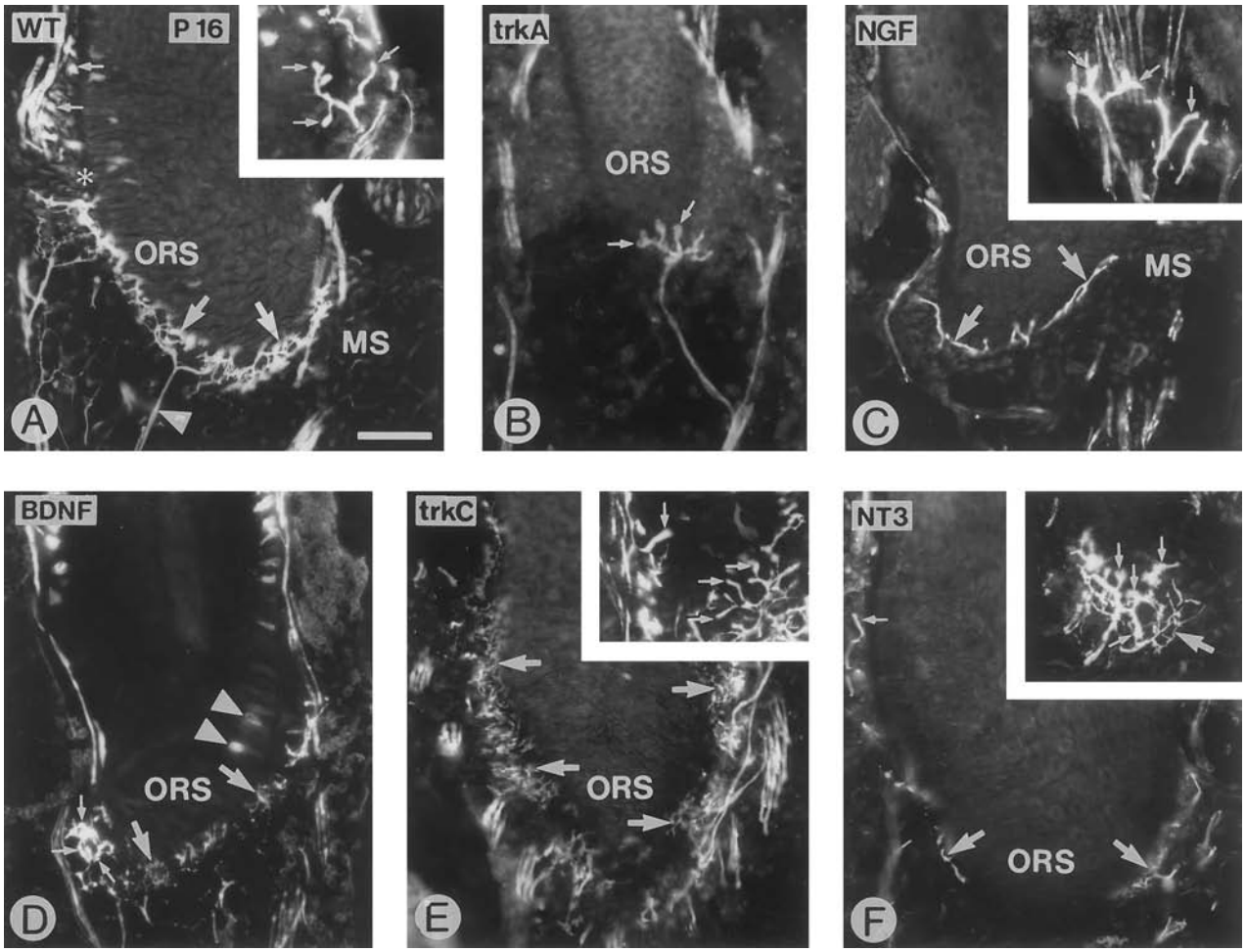



**FIG. 8.** Photomicrographs of the impact of various null mutations on reticular endings and Ruffini endings in the mesenchymal sheath (MS) at the level of cavernous sinus, as revealed with anti-PGP 9.5 in 2-week-old mice. Scale bar, 50 $\mu$m. (A) In the wild-type mouse, medium caliber axons (arrowhead) branched extensively to form numerous reticular endings (large arrows) in the upper portion of the cavernous sinus and in close proximity to the glassy membrane (asterisk). The insert shows Ruffini endings (small arrows) which were located centrifugally to the reticular endings. (B) In the absence of trkA, reticular endings were not present postnatally but Ruffini endings (arrows) were not affected. (C) In the absence of NGF, a few reticular axons survived and formed reticular endings (large arrows) but with considerably fewer branches than normal. As in the trkA null mutation, the Ruffini endings (insert, small arrows) were not affected. (D) Lack of BDNF did not obviously affect the reticular endings (large arrows), whereas only occasional Ruffini endings (small arrows) were evident. Note the increase of Merkel cells at the level of cavernous sinus (arrowheads). (E) A substantial increase in reticular endings (large arrows) was evident in the trkC$^{-/-}$ mice. Ruffini endings were also increased (insert, small arrows). (F) In the absence of NT-3, reticular endings (large arrows) were much less branched at the target, although more than that seen in NGF$^{-/-}$ mice (C). In contrast, Ruffini endings (insert, small arrows) were considerably increased but located closer to the glassy membrane than normally seen in wild-type mice.

The Ruffini endings were completely missing without trkB (not shown), whereas rare endings having a normal morphology were still evident in the absence of BDNF (small arrows in Fig. 8D). Although trkB is the high-affinity receptor for NT-4, the Ruffini endings were not noticeably affected by the absence of NT-4. In contrast to the striking absence of reticular endings at the same location in the FSCs of NGF and trkA knockouts (see below), the Ruffini endings were clearly present in both of these mutants (insert in Fig. 8C, small arrows; Fig. 8B, small arrows).

In contrast to the loss of Ruffini innervation in BDNF and trkB knockouts, far more endings having Ruffini-like characteristics were present than normal in NT-3$^{-/-}$ (insert in Fig. 8F, small arrows) and to a lesser extent in trkC$^{-/-}$ mice (insert in Fig. 8E, small arrows). Although they clearly arose from relatively large caliber axons, these endings were

more branched and were located closer to the basement membrane of the vibrissal follicle than normal.

### *Reticular and Transverse Lanceolate Ending Dependency on NGF, NT-3, and trk, but Not trkC*

In contrast to the Ruffini afferents that also terminate in the mesenchymal sheath of vibrissal FSCs at the upper end of the cavernous sinus, reticular axon and endings are severely depleted in the absence of trkA and to a lesser degree NGF (Fig. 8). Although the endings are reduced without NT-3, they are hypertrophied in trkC$^{-/-}$ mice.

On the day of birth in wild-type mice, only the source axons from DVNs as well as a few immature reticular endings were present at their target in the mesenchymal sheath at the upper end of the cavernous sinus in vibrissal FSCs. These reticular axons branched extensively during the first postnatal week and continued to mature through the second. By the end of the second week, a dense grid of mature well-branched reticular endings emanating from medium caliber axons surrounded each vibrissal follicle (Fig. 8A, large arrows).

In trkA$^{-/-}$ mice, the reticular axons and endings were completely absent at all ages studied (Fig. 8B). In contrast, a few reticular axons with an extremely reduced branching were present in the NGF$^{-/-}$ mice (Fig. 8C, large arrows). A week after birth, absence of NGF resulted not only in fewer reticular afferent axons but also in abnormal ending morphology (not shown).

In addition to the effects obtained by absence of NGF or trkA, reticular endings were decreased after homozygous NT-3 null mutations (large arrows in Fig. 8F). Paradoxically, they increased dramatically in the absence of trkC (large arrows in Fig. 8E). The decrease observed in the NT-3$^{-/-}$ mice at all ages was clearly due to considerably less branching of the axons close to the target rather than to any obvious decrease in the number of axons. Although we could not rule out the presence of additional axons, the increase of reticular endings observed in trkC$^{-/-}$ mice was largely due to an excessive branching of the axons close to the target (Fig. 8E).

Null homozygous mutations of the genes encoding for BDNF, NT-4, trkB, or p75 had no obvious qualitative effect on the reticular endings. Furthermore, no delay or difference in timing of reticular ending postnatal development could be established in any of the affected mutants.

Another set of medium caliber myelinated afferents that are severely depleted in the absence of NGF or trkA are the transverse lanceolate endings that arise *en passant* from SVN axons to the inner conical body of vibrissal FSCs. In contrast to the reticular endings, neither the endings nor their axons were normally present in the FSCs at birth. In wild-type mice their axons entered the FSCs and began to innervate the inner conical bodies between P2 and P3. Unlike the reticular endings, the transverse lanceolate endings in the inner conical body were completely missing not only in trkA$^{-/-}$ mice (Figs. 5E and 6B) but also in the absence of NGF (not shown).

Consistent with the increase in reticular innervation, trkC$^{-/-}$ mice also had a substantial increase in transverse lanceolate innervation in the inner conical body, as evidenced by more RT97-labeled profiles descending from the SVNs and distributing circumferentially (Fig. 3F, small arrows) compared to that in the wild-type mice (Fig. 3B, small arrows). Whether the increase was due mainly to an increased branching of the transverse lanceolate afferents was not obviously apparent because the intermingled axons and endings cannot be distinguished from each other at the light microscopic level (Figs. 3E, 3F, and 6D). As described earlier in regard to the loss of Merkel endings, some medium to large caliber axons ascend abnormally from the DVN in the trkC$^{-/-}$ mice and may partly contribute to the increased RT97-labeled profiles in the inner conical body. NT-3 null mutations also resulted in an increase in RT97-labeled profiles in the inner conical body. However, in this case, most if not all of the increase may be due to the greater number of abnormally ascending DVN axons (long, small arrow in Fig. 3I and arrowhead in Fig. 6E) than occurs after the loss of trkC. BDNF, NT-4, trkB, and p75 null mutations had no obvious effects on the transverse lanceolate endings.

## DISCUSSION

### *Phenotype-Related Dependency upon Specific Combinations of Receptors and Neurotrophins*

Our results demonstrate several major concepts about the role of neurotrophins in the development of cutaneous mechanoreceptors that are supplied by medium to large caliber myelinated afferents:

1. Each of the high-affinity tyrosine kinase receptors, trkA, trkB, trkC, as well as the low-affinity p75 receptor, has an impact on at least one type of morphologically distinct mechanoreceptor supplied by a medium to large caliber myelinated afferent.

2. The elimination of NGF, BDNF, and NT-3 has a similar impact to the absence of their high-affinity receptors: trkA, trkB, and trkC respectively. However, there are also differences consistent with possible NT-3 signaling through its lower affinity for trkA and trkB and by NT-4 binding to trkB (Cordon-Cardo *et al.*, 1991; Ip *et al.*, 1992; Klein *et al.*, 1991b, 1992; Soppet *et al.*, 1991; Squinto *et al.*, 1991). Elimination of NT-4 by itself had no detrimental impact.

3. Each type of afferent is dependent upon a unique combination of neurotrophins and neurotrophin receptors that can involve multiple trophins and receptors acting simultaneously on some afferents or sequentially on others.

4. Regardless of the particular neurotrophin/receptor dependency for afferent survival and neurite outgrowth, NT-3 has an impact on the formation of all the sensory endings.

In contrast, NGF/trkA signaling appears to mediate primarily afferent survival and neurite outgrowth but does not seem to be a major factor in the formation of any sensory endings.

5. BDNF plays a major role in the formation of sensory endings in trkB-dependent phenotypes in addition to supporting the survival of the neuron.

6. The formation of Merkel endings is dependent upon NT-3 signaling through a collaboration of trkA, trkC, and possibly p75. However, continued survival of Merkel innervation becomes completely dependent on NT-3/trkC signaling with long-term dependency on p75.

7. NT-4 and BDNF may act through trkB to suppress Merkel innervation, whereas NT-3 may act through the trkC receptor to suppress Ruffini innervation.

### *Unique Dependencies of Various Mechanoreceptors on Neurotrophins and Receptors*

***TrkA-dependent phenotypes: Reticular and transverse lanceolate afferents.*** In the FSCs of NGF and trkA null mutants, reticular endings and transverse lanceolate endings as well as their source axons were severely depleted or completely absent, at least during the postnatal period examined in our analysis. Thus, these two sets of medium caliber myelinated afferents must pass through a phase in which the existence of their axons is completely dependent on NGF/trkA signaling. This is the first direct evidence that NGF and trkA are essential for the development of some types of medium to large caliber myelinated cutaneous mechanoreceptors.

Since reticular axons are normally present and are just beginning to form their endings at birth, we do not know if these axons ever reached the vicinity of their target—i.e., the mesenchymal sheath—in the absence of NGF or trkA and subsequently degenerated. In the case of the transverse lanceolate innervation, which normally begins to differentiate only 3 days after birth, it was clear in the NGF and trkA knockouts that their axons were not in the vicinity of their target—i.e., the inner conical body—at least between birth and P2. This indicates that the transverse lanceolate afferents are dependent upon NGF/trkA signaling prior to the time when their axons have arrived at their definitive targets.

Our type of analysis could not determine whether the absence of the reticular and transverse lanceolate axons was due to or resulted in the loss of the neuronal cell bodies. However, such a loss would be consistent with the observation that neuronal apoptosis is so high in the dorsal root ganglia (DRGs) and trigeminal ganglia of NGF and trkA knockouts that at least some large sensory neurons are lost in addition to the extensive loss of smaller neurons (Crowley *et al.*, 1994; Ritter *et al.*, 1991; Silos-Santiago *et al.*, 1995; Smeyne *et al.*, 1994; Davis, unpublished). Interestingly, the transverse lanceolate innervation was completely lacking in both the NGF and trkA null mutants, whereas all reticular innervation was lost in the absence of trkA but some survived without NGF. Since the reticular innervation develops a few days earlier, perhaps some of these neurons are generated at a relatively earlier age prior to a dependence on neurotrophins or prior to the availability of NGF (Buchman and Davies, 1993). In the latter case, the surviving neurons or their precursors might have been rescued through trkA signaling as a result of binding with NT-3, which is present relatively early in development (Arumäe, 1993; Cordon-Cardo *et al.*, 1991; ElShamy and Ernfors, 1996a,b; Fagan *et al.* 1996; Klein *et al.*, 1991b; Soppet *et al.*, 1991; Squinto *et al.*, 1991; White *et al.*, 1996; Wilkinson *et al.*, 1996).

Our results indicate that a separate phase of neurotrophin/receptor signaling may be involved in promoting formation of sensory endings at the target. Many if not most of the reticular afferents are present at birth in NT-3 null mutants but they do not form normal endings. This indicates that NT-3 plays an important role in the formation of endings by reticular afferents. Surprisingly, the elimination of trkC caused a paradoxical increase in branching of reticular endings. The loss of trkB had no obvious effect. Combined, these results indicate that NT-3 is signaling through trkA to promote reticular ending formation. The unexpected hypertrophy of reticular endings in the absence of trkC may be due to the fact that trkC knockouts lose adjacent sets of Merkel endings that might otherwise be competing for NT-3 (see below and Fig. 10).

We could not determine whether the absence of NT-3 was also detrimental to transverse lanceolate endings in the inner conical body because their source axons in NT-3$^{-/-}$ mice were intermingled with numerous aberrant axons that ascended abnormally from the DVN (Fig. 10). Since only a few fine caliber DVN axons normally ascend to that level, the identity of these ascending axons is unknown. Possibly they are DVN Merkel afferents whose endings have degenerated at the level of the ring sinus (Fig. 10). Like the reticular innervation, an increase in transverse lanceolate innervation was evident in trkC$^{-/-}$ mutants which have fewer aberrant axons ascending from the DVN. This indicates that the formation of transverse lanceolate endings may also be dependent upon NT-3 signaling through trkA.

In summary, our results indicate that reticular and transverse lanceolate afferents may be types that express trkA both early and late during their development. At earlier stages, trkA, primarily by binding with NGF, may be involved in the survival of the neurons and/or outgrowth and survival of their axons. The survival of some of the reticular afferents may also occur through NT-3/trkA signaling. At later stages, NT-3/trkA signaling may be a major contributor to the formation of the endings.

***TrkB-dependent phenotype: Ruffini afferent.*** The Ruffini endings were the only ones completely eliminated by trkB null mutations. Because of the close proximity of numerous intact reticular afferents in trkB knockouts, we could not determine definitively whether the absence of Ruffini endings was limited to reduced formation of these relatively sparse endings or if the source axons were elimi-

nated. Although this innervation was severely depleted in the absence of BDNF, occasional endings still existed indicating that these afferents may be partially supported by other neurotrophins, such as NT-4 or NT-3, which also bind to trkB and are both expressed in the outer root sheath during development (Glass *et al.,* 1991; Ibáñez *et al.,* 1993; Ip *et al.,* 1992; Klein *et al.,* 1994; Soppet *et al.,* 1991; Squinto *et al.,* 1991). Conceivably, the Ruffini afferents may be among the larger size neurons that are lost in the trigeminal ganglion subsequent to trkB or BDNF null mutations (Ernfors *et al.,* 1994a; Jones *et al.,* 1994; Klein *et al.,* 1993) and may therefore be among those developing neurons who express high levels of trkB (Arumäe, 1993; Wright and Snider, 1995; Snider and Wright, 1996).

The elimination of NT-4 did not cause a noticeable change in the Ruffini innervation, suggesting that the presence of BDNF alone may be sufficient to support their development without a contribution from NT-4. Thus, the Ruffini afferents do not appear to be among the sets of neurons that are lost in the absence of NT-4 (Conover *et al.,* 1995; Liu *et al.,* 1995).

As occurred among reticular and transverse lanceolate afferents, Ruffini innervation was increased in the absence of trkC although this increase appears to involve both the number of axons and their branching. However, in striking contrast, the Ruffini innervation was increased even more in NT-3$^{-/-}$ mice instead of being reduced. This suggests that an NT-3/trkC signaling mechanism may exist that could be having a suppressing effect on the Ruffini innervation. Thus, Ruffini afferents may express trkB and trkC either simultaneously or sequentially at some phase during their development but the signaling through these receptors may have opposing effects.

Alternatively, the absence of NT-3 could be hastening the differentiation of neuronal precursors that consequently become contributors to the Ruffini afferent population (Fariñas *et al.,* 1996) at the expense of other populations such as Merkel afferents (see below).

***TrkA-, trkC-, and p75-dependent phenotype: Merkel afferents.*** In addition to confirming the dependency of Merkel innervation on NT-3 (Airaksinen *et al.,* 1996), our results indicate that this dependency occurs by NT-3 signaling primarily through the trkC receptor. However, these afferents also appear to have complex neurotrophin dependencies that may involve NGF, trkA, and p75 and may be suppressed by NT-4 and BDNF signaling through trkB. These conclusions are based on several observations discussed below.

The loss of Merkel innervation occurs earlier in NT-3$^{-/-}$ mutants than in those lacking trkC. Since the loss of Merkel innervation is so severe in the newborn NT-3$^{-/-}$ mice, much of it may not even begin to form during the prenatal period in the NT-3 homozygous mutants. Assuming that the loss of Merkel innervation is accompanied by an increase in apoptosis, the discrepancy in timing may contribute to the higher cell loss observed in ganglia from NT-3$^{-/-}$ compared to trkC$^{-/-}$ newborn mice (Ernfors *et al.,* 1994b; Fariñas *et al.,* 1994; Klein *et al.,* 1994; Tessarollo *et al.,* 1994). Comparable counts were not made at later ages, after the Merkel innervation has deteriorated in the absence of trkC.

Presumably, the relatively prolonged survival of Merkel innervation in the trkC knockouts may be due to NT-3 signaling through a coexpressed receptor such as trkA. In fact, our observations indicate that some Merkel innervation is dependent entirely upon NGF/trkA signaling at some phase during their development. In particular, Merkel cell–neurite complexes at lower levels of the ring sinus were eliminated in trkA$^{-/-}$ as well as NGF$^{-/-}$ mice. Consistent with these observations, preliminary results indicate that the entire Merkel innervation is missing at birth in trkA$^{-/-}$/trkC$^{-/-}$ hybrid mice (Rice, Fundin, and Silos-Santiago, unpublished) and that anti-trkA immunoreactivity is expressed on Merkel endings in prenatal rats (Jhaveri and Rice, unpublished). Also, trkC mRNA is coexpressed with trkA mRNA in many developing neurons in dorsal root ganglia (White *et al.,* 1996) and trigeminal ganglia (Silos-Santiago, unpublished observation). Thus, trkA receptors may be present in many Merkel afferents in sufficient amounts for NT-3 to sustain their early survival in the absence of trkC, but these coexpressed trkA receptors may not be essential for survival in the presence of intact trkC. Such an effect was observed among many neurons in developing dorsal root ganglia (White *et al.,* 1996).

Consistent with a NT-3-dependent prenatal loss of Merkel innervation, NT-3$^{-/-}$ mice have been shown to have a major reduction in trigeminal neurons early in development, although the basis for this decrease is controversial. One study indicates that the loss is primarily due to apoptosis of precursor cells (ElShamy and Ernfors, 1996b), while another indicates that mostly postmitotic immature neurons are degenerating (Wilkinson *et al.,* 1996). In the former study, the normal source of NT-3 support for precursor cell survival is attributed to local cells within and near the ganglion. In the latter study, normal NT-3 support for the neuronal survival is attributed primarily to their target, such as the mystacial pad. Another contributing factor to the NT-3$^{-/-}$ related absence of Merkel innervation could be a reduction of proliferating precursor cells due to premature differentiation (Fariñas *et al.,* 1996).

Although trkA may contribute to early Merkel afferent survival, Merkel innervation that survives the absence of trkA continues to be maintained as long as 4 weeks postnatally, which was the oldest trkA$^{-/-}$ specimen examined. In contrast, all of the Merkel innervation is lost by the end of the second week in the absence of trkC. This indicates that only NT-3/trkC signaling is essential for short-term maintenance. Consistent with this observation, anti-trkA immunoreactivity was not observed on Merkel endings in neonatal rats (Jhaveri and Rice, unpublished), indicating that the prenatal expression of trkA may become downregulated near the end of gestation.

Interestingly, the loss of NGF, but not trkA$^{-/-}$, resulted in an increased among the subsets of Merkel innervation

9

ⓐ **Transverse lanceolate endings**

↓ **NGF, trkA**

↑ NT3, trkC

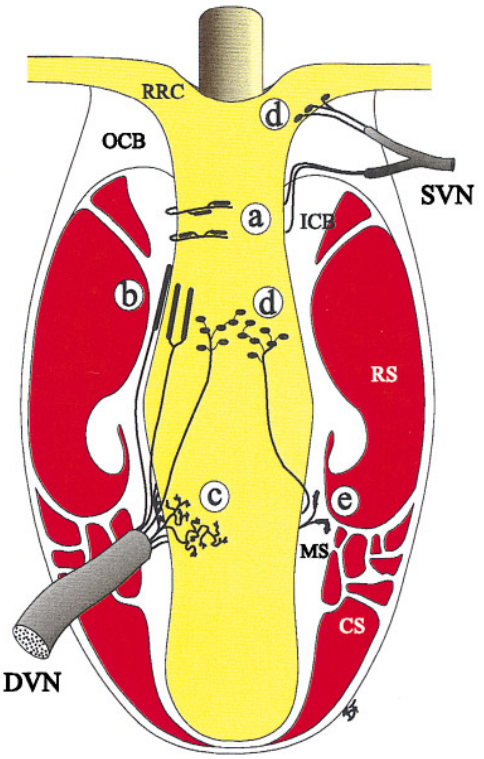
ⓑ **Longitudinal lanceolate endings**

↓ NGF, trkA
BDNF, trkB

ⓒ **Reticular endings**

↓ NGF, NT3, **trkA**

↑ trkC




ⓓ **Merkel cell-neurite complexes**

↓ **NT3, trkC**
NGF, trkA, p75

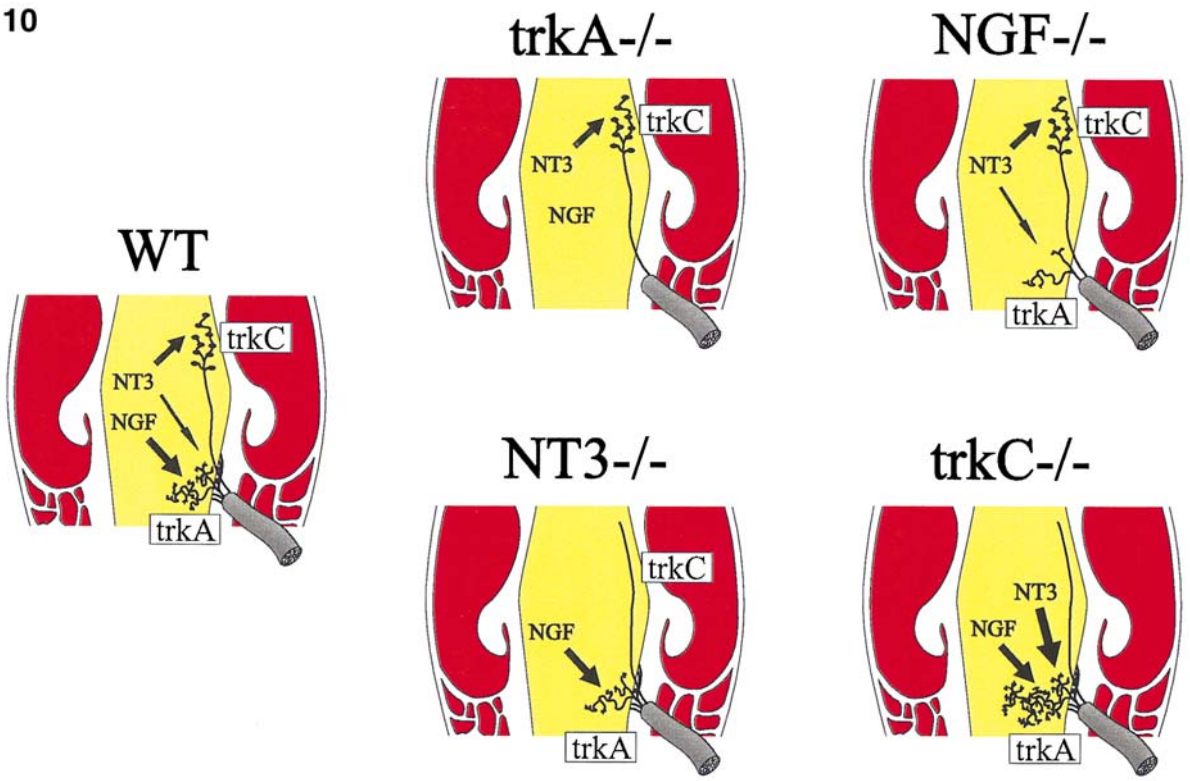
↑ BDNF, NT4, trkB
p75 (Merkel cells)

ⓔ **Ruffini endings**

↓ BDNF, **trkB**

↑ NT3, trkC

10



in the epidermis at the mouths of vibrissal and guard hair follicles. In this particular case, the loss of NGF eliminates several sets of intermingled unmyelinated epidermal innervation that are totally dependent upon NGF for their survival but also use NT-3/trkA signaling for the formation of their endings (Rice and Fundin, unpublished observation). Thus, a loss of competition for NT-3 may result in an increase of NT-3/trkC signaling among the local epidermal Merkel innervation. Consistent with this hypertrophy and the role of NT-3 in the development and maintenance of these afferents, Merkel innervation is increased in the epidermis of mice that have sustained high levels of NT-3 produced in keratinocytes as a result of transfections with constructs consisting of NT-3 cDNA linked to a keratin promoter gene (Albers *et al.*, 1996).

One puzzling aspect of the NT-3 and trkC null mutations is the loss of Merkel cells, or at least Merkel cell labeling, shortly after the disappearance of their innervation. In contrast, Merkel cells at the level of ring sinus remain at least 2 weeks following neonatal nerve transection (Nurse and Farraway, 1988) and are detected with anti-PGP 9.5 and anti-CGRP immunofluorescence even longer following nerve transection in the adult (Rice *et al.*, 1993; Fundin and Rice, unpublished). These observations raise the possibility that the Merkel cells themselves may be dependent upon NT-3/trkC signaling during the perinatal and early postnatal periods. Conceivably, the loss of Merkel endings might be due to deterioration beginning in the Merkel cells which subsequently may not be able to sustain their innervation. For example, Merkel cells have been shown to produce NGF *in vitro* which can support sensory neurite outgrowth (Vos *et al.*, 1991). In view of the possibility that the maintenance of Merkel endings may be dependent on the Merkel cells, the DVN axons that ascend abnormally to the inner conical bodies in the FSCs of NT-$3^{-/-}$ and trk$C^{-/-}$ mice might be surviving Merkel axons that have lost their endings. Alternatively, these axons may be from afferents that have undergone a phenotypic switch in the absence of NT-3 or trkC. Whether they form functional terminations in the inner conical body remains to be determined and may have important consequences for the analysis of mechanoreceptor physiology.

After 2 postnatal weeks in $p75^{-/-}$, Merkel endings gradually become depleted over a period of several weeks but the Merkel cells remain evident. This result may be a reflection of two different roles for the p75 receptor. For the maintenance of Merkel endings, p75 may facilitate the NT-3/trkC mechanism as levels of NT-3 become reduced during maturation. Several studies have shown that the p75 receptor can facilitate neurotrophin binding to high-affinity receptors, increase uptake and transport of neurotrophins, and lower response thresholds to neurotrophins (Barker and Shooter, 1994; Benedetti *et al.*, 1993; Curtis *et al.*, 1995; Hantzopoulos *et al.*, 1994; Lee *et al.*, 1994a,b; Rydén *et al.*, 1995; Verdi *et al.*, 1994). For the prolonged survival of the denervated Merkel cells, the lack of $p75^{-/-}$ may have prevented the activation of a p75-mediated apoptotic mechanism (Barrett and Bartlett, 1994; Frade *et al.*, 1996; Rabizadeh *et al.*, 1993).

In contrast to the detrimental effects of the NT-3, trkC, and p75 deletions, the null mutations of NT-4, BDNF, or trkB resulted in an increase in Merkel innervation in all locations. These observations suggest that an NT-4 and BDNF signaling mechanism through the trkB receptor may have a suppressing effect on the Merkel innervation, perhaps by causing a downregulation of the trkA receptor (Wyatt and Davis, 1993). This would suggest that the trkB receptor may also be expressed by the Merkel neurons together with trkA and/or trkC (Snider and Wright, 1996). A similar trkB mechanism also appears to suppress several sets of unmyelinated epidermal innervation (Rice and Fundin, unpublished observation).

In summary, Merkel afferents may be a type of neuron that could conceivably express all three high-affinity trk receptors and the low-affinity p75 receptor, at least at some phase during their development. In view of observations

**FIG. 9.** Schematic drawing summarizing the mechanoreceptor response to the different neurotrophin and receptor null mutations. The type of ending is indicated with boldface letters, with their response listed below. Null mutations which had a deleterious impact on the mechanoreceptor population are indicated with down arrows. Null mutations resulting in obvious increases among mechanoreceptor populations are indicated with up arrows. Bold print indicates that the set was totally eliminated. The lowercase letters that proceed each ending type correlate with the letters shown in the drawing that indicate their normal localization in the FSC.

**FIG. 10.** Schematic drawing illustrating the suggested competition of mechanoreceptor afferents on different neurotrophins at the target tissue during development and how that may account for the results obtained in the different null mutations. In wild-type (WT), reticular afferents, which terminate at the level of cavernous sinus, require NGF/trkA signaling to survive but also need NT-3/trkA signaling to proliferate normal endings. Merkel cell–neurite complexes at the level of ring sinus are dependent on NT-3/trkC signaling for their maintenance. TrkA and trkC receptors, which presumably are expressed on reticular endings and Merkel endings, respectively, should normally bind and occupy the NGF and NT-3 which has been shown to be produced by the follicle cells during development (indicated with arrows). In the absence of trkA, reticular afferents are completely missing. A few reticular afferents survive in NGF knockouts but branch poorly. This partial survival in the absence of NGF might be due to NT-3/trkA. The decreased branching of reticular endings in the absence of NT-3 indicates that NGF/trkA signaling alone is not sufficient for normal development. The loss of Merkel afferents in the absence of trkC may result in less competition for NT-3, which in turn may lead to an increase in the branching of reticular afferents.

that Merkel neurons may be generated over a relatively long period of time and distribute to different locations at different times (see Nurse and Farraway, 1988; Pasche *et al.*, 1990), particular subsets may have somewhat different receptor pedigrees even though they form a phenotypically similar type of ending. For example, most Merkel afferents appear to be dependent only on NT-3 signaling through trkC and trkA for their prenatal survival, whereas as some may depend only upon NGF/trkA signaling. During the early postnatal period, all of the Merkel innervation depends solely on NT-3/trkC signaling for their continued maintenance. For their long-term maintenance they require p75. In contrast, BDNF and NT4 signaling through trkB may have a suppressing effect.

***TrkA- and/or trkB-dependent phenotype: Longitudinal lanceolate afferents.*** The longitudinal lanceolate endings in the mesenchymal sheath of vibrissal FSCs were substantially depleted by trkB null mutations and to a lesser extent by the elimination of trkA. The absence of each of either receptor also causes a substantial loss of longitudinal lanceolate endings in guard hair piloneural complexes. BDNF and NGF null mutations caused proportionately similar deficits but neither was as severe as the loss of the corresponding trkB and trkA receptor. In both cases, the decrease appears to include a loss of axons as well as endings, suggesting that these receptors play a role in the possible outgrowth and/or the survival of the afferents. The less severe impact of the loss of these neurotrophins may be due to the fact that NT-4 can also bind to trkB and NT-3 to trkA and trkB. However, the NT-4 null mutation had no noticeable deleterious impact, suggesting that NT-4 may normally be insufficiently available or does not have an additive developmental effect in the presence of BDNF. Thus, the absence of at least some axons may be related to the increased loss of larger size ganglion cells that occurs in trkB as well as trkA knockouts (Klein *et al.*, 1993; Smeyne *et al.*, 1994), but not in NT-4 knockouts.

Interestingly, the morphology of the longitudinal lanceolate endings that survived after NGF or trkA knockouts differed from those surviving after BDNF or trkB knockouts. The former were short and had several branches, while the latter were long and had only one or two branches. Normal FSCs contain a mixture of both types. Thus, NGF/trkA signaling appears to support the long ending phenotype, whereas BDNF/trkB signaling supports the short. These results, along with only the partial loss of lanceolate afferents after deletion of either neurotrophin or either receptor, suggest two alternative hypotheses. On the one hand, there may be two distinct lineages of lanceolate afferents: a long ending type that only expresses trkA and is dependent on NGF/trkA signaling and a short ending type that only expresses trkB and is dependent on BDNF/trkB signaling. Alternatively, there may be only one lineage of lanceolate afferent progenitors that gradually shift from trkA to trkB expression (or vice versa) over the entire period of their neurogenesis. Thus, the proportion of coexpression of trkA and trkB may skew a particular afferent toward a greater dependency on one receptor or the other for both their survival and morphological expression of their ending. Relative to either hypothesis, trkA and trkB mRNAs have been shown in separate and overlapping populations in dorsal root ganglia (Snider and Wright, 1996; Wright and Snider, 1995).

These alternative hypotheses raise an important difference about the potential role of the neurotrophins in the development of sensory endings. If there are indeed two separate lineages, then each may already be preprogrammed for the type of ending they will form. Therefore, the particular signaling pathway may be important for survival and ending formation but might not be a factor in regulating the morphology of the ending. If the second hypothesis is correct then the signaling pathway may be important for regulating the ending morphology. Consistent with the latter hypothesis, the elimination of NT-3 does not cause a noticeable reduction in lanceolate innervation but does shift the morphology to a shorter more highly branched form.

The apparent codependency of longitudinal lanceolate afferents on NGF and BDNF might partly be due to a combination of a direct BDNF/trkB effect and an indirect NGF/trkA effect. As shown recently by Robinson *et al.* (1996), NGF/trkA-dependent neurons in the developing dorsal root ganglion are a source of BDNF, which may in turn promote the survival of BDNF-dependent neurons through a paracrine interaction. Consequently, in NGF or trkA knockouts, the loss of NGF/trkA-dependent neurons might in turn result in the elimination of many BDNF/trkB-dependent lanceolate afferents. The lack of a complete elimination of lanceolate afferents by any one of the NGF-related neurotrophins or receptors might also be due to support through other trophic factors, such as glial-derived neurotrophic factor (Buj-Bello *et al.*, 1995).

## *Other Impacts of p75 Null Mutations*

In addition to the impact on the Merkel innervation, the p75 elimination resulted in a transient loss of RT97 labeling in the distal portion of all types of DVN mechanoreceptor axons between birth (when RT97-IR is initially throughout the axons) and the fourth postnatal week (when RT97-IR is restored distally). During the intervening period, RT97 immunoreactivity is limited to proximal portions of the axons in the infraorbital nerve fascicles. Presumably this indicates that at least the 200-kDa phosphorylated neurofilament protein subunit is transiently absent distally. Whether this loss has a functional impact on this innervation remains to be elucidated. In contrast to the impact on DVN afferents, some RT97-IR continues to persist in the SVNs. The differences between RT97-IR in the DVN and SVN axons might be related to the observation that the transport of BDNF, NT-4, and NT-3, but not NGF, are dependent upon the p75 receptor (Curtis *et al.*, 1995; von Bartheld *et al.*, 1994). The SVNs supply the only mechanore-

ceptor—transverse lanceolate endings—that are completely eliminated by the loss of NGF.

### *Impact on Barrel Formation*

In other studies of the trigeminal brainstem nuclei in the various trk knockout mice, vibrissa-related barrel patterns have been shown to develop at least somewhat normally (Henderson *et al.*, 1995) despite the loss of innervation in the related mystacial pad. This result may be due to the fact that none of the trk receptor deletions eliminates all of the vibrissa-related mechanoreceptors. Instead, each eliminates particular types of mechanoreceptors. Previous physiological studies combined with intra-axonal injections have demonstrated that several varieties of vibrissa-related mechanoreceptors project to each vibrissa-related site in the brainstem (Jacquin *et al.*, 1986, 1992, 1993). Thus, none of the trk deletions would result in a complete denervation of a vibrissa-related site. The remaining afferents may be sufficient to organize the barrel patterns.

### *General Conclusions*

Overall, our study demonstrates that NGF/trkA, BDNF/trkB, and NT-3/trkC signaling as well as signaling involving the p75 receptor all have an impact on the development or maintenance of at least one specifically identifiable type of cutaneous mechanoreceptor. In turn, each type of afferent is dependent upon a unique combination of receptors and neurotrophins. In some afferents, the combination may involve a single neurotrophin and receptor. Others may use multiple neurotrophins and/or receptors. Thus, NGF/trkA is important in the development of many larger myelinated types of cutaneous afferents as well as unmyelinated afferents and postganglionic sympathetic neurons. Also, NT-3 apparently can signal through trkA, trkB, and trkC to prevent some apoptosis among sensory ganglion cells, but plays a major signaling role through all three receptors for the formation and/or maintenance of the sensory endings. On some afferents, neurotrophin signaling through a trk receptor may have a suppressing effect, as evidenced by increased innervation as a result of deletions of either the neurotrophin or the neurotrophin receptor. In other cases, the innervation density may be determined by competition for the same neurotrophin by different sets of afferents. Thus, the overall distribution of the various sets of sensory innervation appears to be due to a balance of differential dependencies upon the various neurotrophins, of suppressing as well as enhancing signaling mechanisms, and by competition for the same neurotrophins by different sets of afferents.

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